THE ANAEROBIC DECOMPOSITION OF BENZOIC ACID DURING METHANE FERMENTATION: EVIDENCE THAT CARBON FOUR OF BENZOIC ACID IS CONVERTED TO THE CARBOXYL OF THE INTERMEDIATE PROPIONIC ACID

by

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INTRODUCTION

The biological breakdown of aromatic compounds and substances such as lignin, dyes, detergents and industrial wastes which contain aromatic type compounds is an integral part of nature's complex ecological system. This process is accomplished by several kinds of microorganisms; however, bacteria are the most versatile in this respect.

Anaerobic methane producing bacteria may play a very important role in the decomposition of certain of these aromatic compounds; however, the mechanism whereby this occurs is rather obscure. Little attention has been given to the anaerobic utilization of aromatic compounds by these bacteria because of strict anaerobic requirements, relatively slow rate of growth and technical difficulties. Another reason for lack of interest in this group of bacteria might have been the general belief that microorganisms were not capable of attacking ring compounds in the absence of elemental oxygen.

Methanogenic bacteria are present wherever there is natural microbial decomposition of organic material under anaerobic conditions. Two places where anaerobic decomposition of aromatic compounds are of prime importance is in the rumen and the digestors of municipal sewage systems. The significance of methane bacteria in the overall picture of aromatic decomposition is not clear but it would appear that they are a factor in returning aromatic type materials to inorganic compounds.

In this investigation, an isolated system was used to study the utilization of benzoic acid as a sole carbon and energy source. This model system was comprised of cultures of methanogenic bacteria in all-glass fermentors equipped with a manometer and stopcocks. A series of experiments were set up to study the fate of benzoic acid species labeled in various positions with ^{14}C. This study was undertaken to add information or further delineate a possible anaerobic aromatic pathway.

REVIEW OF LITERATURE

It has been acknowledged for many years that methane is characteristically produced by microbial decomposition of organic compounds under anaerobic conditions. In Roman times, Pliny described the escape of combustible gases from the earth's surface; and as early as 1776, Volta recognized a relationship between the amount of gas evolved and the amount of plant material in lake sediments (Barker, 1956). Many other early examples may be cited; however, only recently have the metabolic pathways of these elusive methane bacteria been investigated.

The methane bacteria have been difficult to characterize because of the problems involved in growing, isolating and maintaining pure cultures. In fact, it was not until 1936 that attempts to isolate pure cultures or even obtain growth of colonies in solid media were successful (Barker, 1936). Because of these difficulties even today, most studies with methane bacteria are carried out with highly enriched cultures.

All bacteria that produce methane are strict anaerobes and they will proliferate only in the absence of oxygen and in the presence of suitable reducing agents. They are much more sensitive to oxygen and other oxidizing agents, such as nitrate, than most other anaerobic bacteria (Barker, 1936). For this reason, they are more easily grown in liquid or semisolid media. Solid sediments, such as diatomaceous earth or shredded asbestos, added to the liquid media to provide mechanical shielding from any dissolved oxygen are beneficial (Breden and Buswell, 1933).

Nutritional requirements of the methanogenic bacteria are relatively simple. Generally, an organic acid plus inorganic mineral salts and the ammonium ion are sufficient for growth and methane production. However, each species has a relatively specific substrate requirement for carbon and energy source: Methanobacterium propionicum requires propionate (Stadtman and Barker, 1951); Methanobacillus omelianskii is specific for primary and secondary short chain aliphatic alcohols (Barker, 1941) and hydrogen (Barker, 1943); Methanococcus mozei utilizes acetate and butyrate (Barker, 1936); Methanosarcina barkerii utilizes methanol, acetate and carbon dioxide (Schnellen, 1947). Many substrates decomposed by bacteria, such as amino acids and carbohydrates, are not attacked by pure cultures of methane bacteria. For decomposition of a more complex substrate such as benzoate, Fina (1950, 1960) reported that more than one species of bacteria may be involved. Generally, once cultures of methanogenic bacteria are established in a laboratory, they can be maintained indefinitely. No toxic by-products are formed and the major

products, carbon dioxide and methane, can easily be removed.

The decomposition and utilization of aromatic compounds is an essential biological chemical step in nature's carbon cycle. This process is accomplished by a variety of microorganisms with bacteria playing the major role. In the majority of the cases of aromatic ring metabolism, molecular oxygen is an obligatory oxidant (Evans, 1963). Bacteria concerned with the aerobic decomposition of aromatic compounds are widespread in nature with a variety of species involved. The consideration of the many compounds and numerous metabolic pathways is beyond the scope of this discussion; however, characteristic of these processes is the oxidation of side chains, when present, and the cleavage of one cyclic unit at a time. Ultimately, the intermediates are oxidized to a benzene derivative and then cleaved to a noncyclic molecule. These ring fission products are then funnelled into the Krebs cycle through a variety of pathways depending on the organism and cultural conditions. The pathway of aerobic degradation of most aromatic structures passes through this stage and catechol and one or two related substances are the last cyclic compounds preceding ring cleavage (Alexander, 1967). For a more comprehensive discussion of oxidative rupture of aromatic compounds, see Evans (1963).

In contrast to the above cases of aromatic ring metabolism, where molecular oxygen is an obligatory oxidant, there exists an anaerobic type of aromatic ring metabolism. Although all known pathways for degradation of aromatic compounds involve the participation of molecular oxygen (Evans, 1963), certain

derivatives of benzene are metabolized under anaerobic conditions.

The first report of anaerobic utilization of an aromatic compound, specifically benzoic acid, was made by Tarvin and Buswell (1934) in their paper on "The Methane Fermentation of Organic Acids and Carbohydrates". They were attempting to determine if the Knoop method of phenyl-labeling was applicable in the study of the degradation of fatty acids by enriched cultures of methane bacteria. Surprisingly, they observed complete destruction and gasification of the ring and side chains. Although this made the proposed method of study useless, it did indicate, for the first time, that the aromatic ring could be attacked anaerobically.

This work was confirmed by Fina (1950) and Clark and Fina (1952) when they demonstrated that benzoic acid could be degraded anaerobically during methane fermentation. This work further showed that a stabilized culture could be made to return predicted volumes of gas simply by varying the amount of benzoic acid fed. In addition they showed, by simultaneous adaptation experiments (Stanier, 1947), that two intermediates, catechol and protocatechuic acid, common to most aerobic aromatic pathways, are not attacked by benzoic acid enriched cultures under anaerobic conditions. This suggested that the anaerobic and aerobic pathways differ.

In an attempt to determine whether the carbons of the benzoic acid ring were first degraded to carbon dioxide and then reduced to methane, as generally accepted during methane fermentation, tracer studies were undertaken. These experiments showed

that very little carbon dioxide was reduced to methane but was formed by an unknown mechanism (Clark and Fina, 1952).

Several significant exceptions to the scheme of oxidation of organic compounds to carbon dioxide and subsequent reduction to methane have been noted in the literature. Buswell and Sollo (1948), using an acetate substrate and carbon-14-labeled carbon dioxide, showed that a very small part of the methane was derived from carbon dioxide. Pine and Barker (1956) using deuterium-labeled acetate confirmed this completely. The entire methyl group of acetic acid was transferred, as an unchanged unit, to methane. Pine and Vishniac (1957) also showed that the methyl carbon of acetic acid and the methyl alcohol carbon are reduced directly to methane.

Fina et al. (1960) felt that the formic acid dehydrogenation to carbon dioxide and subsequent reduction to methane, as well as the exchange phenomenon between carbon dioxide and formate, might not be completely correct. These authors showed by use of $^{14}CO_2$ that a substantial part of the methane results from direct reduction of the formate. And as previously stated, the exchange phenomenon between the formate and the carbon dioxide was not evident.

Fina and Fiskin (1960) were able to demonstrate by tracer studies that carbon-7 of the benzoic acid ring became carbon dioxide while carbon-1 is converted almost entirely to methane when benzoic acid was used as a substrate for enriched cultures of methane bacteria. Further investigations by Roberts (1962), using benzoic acid utilizing cultures of methane bacteria,

revealed that carbon-4 was also converted primarily to carbon dioxide. He also presented evidence that propionic acid isolated from culture fluid liquor might be an intermediate. He found that the propionic acid contained the labeled carbon-4 of the ring but did not contain carbon-1 or carbon-7. From this evidence, Roberts was able to speculate that ring rupture must occur between carbons 1 and 2 or 1 and 6. Other supporting evidence which favors propionate as an intermediate in benzoic acid metabolism was the observation that the benzoic acid adapted cultures evolved methane and carbon dioxide with no adaptive lag when propionate was used as a substrate. This observation is in agreement with the theory of simultaneous adaptation (Stanier, 1947).

MATERIALS AND METHODS

Preparation of cultures. The methanogenic benzoic acid utilizing stock cultures used in this study were being maintained in this laboratory. These stock cultures were first developed from sludge obtained from anaerobic digestors at the Manhattan, Kansas, sewage disposal plant (Roberts, 1962). They are capable of metabolizing benzoic acid as a sole carbon and energy source under anaerobic conditions.

The experimental subcultures used in this study were obtained from stock cultures by inoculation into 500 ml all-glass fermentors. These fermentation flasks were equipped with a manometer for gas measurement and glass stopcocks covered by

serum stoppers as pictured in Plate I. These flasks were prepared by adding a shredded asbestos nidus as suggested by Breden and Buswell (1933), and Barker's mineral salt solution (Barker, 1936) as modified by Fina et al. (1960). (See Appendix for composition of solutions.) This medium was then adjusted, prior to inoculation of bacterial cultures, to a pH of 6.8 to 7 and heated at 100° C while being flushed with oxygen free nitrogen to insure anaerobiosis. The cultures were maintained in a 40° C incubator. Gas produced by the cultures was collected and measured in a calibrated manometer over a saturated solution of lithium chloride (Boell et al., 1939).

Preparation and feeding of substrate. The substrate fed to the cultures was prepared by dissolving benzoic acid in Barker's mineral salt solution A in an amount necessary to make a 0.1 M solution. (See Appendix.) This substrate was then stored under oxygen free tap gas, in the refrigerator, and kept under anaerobic conditions. Feeding of the cultures was accomplished by injecting the desired amount of substrate through the serum stopper and glass stopcocks on the fermentors. Any liquid removed from either the fermentation flasks or the flask containing substrate for feeding was replaced with oxygen free tap gas.

Establishing cultures in carbon balance and steady state. The experimental cultures were placed in carbon balance during the period of investigation and maintained in a steady state of gas production while actual experiments were being carried out. The term, carbon balance, refers to a dynamic state wherein the amount of substrate fed, in terms of atoms of carbon, is equal to

EXPLANATION OF PLATE I

This fermentation flask is representative of the ones used in the investigation. The flasks are equipped with a manometer for gas measurement and all-glass stopcocks covered with a rubber serum stopper for feeding of substrate and removal of culture liquor.

and reflected by the amount of gas evolved as methane and carbon dioxide. That is, one mole of benzoic acid having seven carbon atoms will produce seven moles of gas.

The cultures were placed in a steady state of gas production by feeding benzoic acid substrate at an interval which allowed for an optimum backlog of substrate in the cultures at all times. Cultures placed in steady state are able to produce gas at an almost steady rate. Several days prior to each experiment, the cultures were forced to produce gas at a maximum steady rate by gradually decreasing the feeding interval and increasing the amount of substrate fed.

Radiochemicals. Radioactive chemicals used in this study, except for benzoic-4-^{14}C acid, were purchased from New England Nuclear Corporation, Boston, Massachusetts. Benzoic-4-^{14}C acid is not commercially available. The author was able to use part of the benzoic-4-^{14}C acid graciously given to Dr. Louis R. Fina by Drs. L. M. Henderson[1] and G. P. Mathur of the Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma (Mathur et al., 1964).

Preparation of cultures for testing and collection of culture liquor. Experimental cultures were established in steady state as previously described. At a designated time, each culture was fed benzoic acid labeled in either the 1, 4 or 7 positions containing one to three microcuries of carbon-14.

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[1]Presently Head, Department of Biochemistry, University of Minnesota, St. Paul, Minnesota.

This was accomplished in conjunction with their normal feeding schedule. After approximately 14 hours incubation, using isotopic dilution, two millimoles of neutralized nonlabeled propionic acid were introduced into each culture. After five additional hours of incubation, the culture liquor from each fermentor was completely removed by needle and syringe. Anaerobic conditions were always maintained, using tap gas as previously mentioned, within the fermentors during this process. A total of from 300 to 400 ml of liquor was removed from each fermentor, placed in storage bottles and frozen until further testing.

Re-establishment of cultures after removal of liquor. After removal of liquor, each culture was immediately replenished with Barker's mineral salt solution and placed back in the incubator for stabilization of the culture for future experiments. During the period between experiments, the cultures were assayed at intervals for elimination of radioactivity. This was accomplished by removing one ml of the culture liquor and checking for the level of activity in a Packard liquid scintillation counter. The cultures were considered ready for a new set of experiments when the level of activity dropped to background counts.

Preparation of culture liquor for analysis. From this point on in each experiment, culture liquor samples were individually tested for the incorporation of specific labeled carbon atoms of various benzoic acid species into the suggested propionic acid intermediate (Roberts, 1962). Each sample was transferred to a distillation apparatus, acidified with ortho-phosphoric acid,

distilled and collected under CO_2 free nitrogen gas until about 90% of the sample was recovered. The nondistillate was then flushed with 50 ml of water and distillation was continued to completion. The distillation of the samples removed salts and particulate matter while allowing recovery of any volatile fractions.

The distillate from each culture was then divided into two equal samples for separate analysis. Each pair of samples was then made basic with 5 N NaOH and evaporated under a stream of CO_2 free nitrogen to a volume of three ml. One of the samples was then transferred directly to a reaction flask for the Phares-Schmidt degradation reaction and evaporated to dryness (Phares, 1951). Most of the benzoate was removed by centrifugation after crystallization at pH 4 and 4° C.

The second sample was transferred to a microsteam distillation apparatus. The apparatus and procedure were designed by Fina and Sincher (1959). Ortho-phosphoric acid (85%) was added to the three ml sample and 25 ml were steam distilled into a second degradation reaction flask. The distillate was then titrated with 1 N NaOH using a Gilmont ultra microburet and phenolphthalein (1 gm in 100 ml of 65% ethanol) as an indicator to determine the millimoles of VFAs in the sample. After titration, this sample was likewise evaporated to dryness. During titration, a stream of CO_2 free air was bubbled through the solution. The CO_2 free air was obtained by using an Oscar air pump to force air through a train consisting of a concentrated liquid KOH solution, solid NaOH and solid $CaSO_4$ (anhydrous

drierite) (Chen, 1957).

Identification of the propionic acid intermediate. A small quantity of each dried sample was analyzed on an Aerograph Model 600 C gas chromatograph to confirm the presence and purity of propionic acid in the sample.[1] A 6 ft x 1/8 in copper, polyethylene glycol on 60-80 mesh Chrom Z column was used. The temperature was 110° C with a gas flow rate of 50 ml/min.

The dried sample was dissolved in a minimal quantity of water, acidified with 85% ortho-phosphoric acid and the water was absorbed and removed by anhydrous Na_2SO_4. Fifteen ml of hexane was then added and evaporated to less than 1/4 ml for injection into the chromatograph. Hexane had previously been determined as a suitable solvent.

Degradation of the propionic acid intermediate. All samples were degraded by the Phares-Schmidt procedure, described by Phares (1951), with several modifications. In the first series of experiments, the carbon dioxide from the decarboxylation of the propionic acid in the sample was trapped in ten ml of 0.5 N NaOH as suggested by Phares (1951). In the second series of experiments, the carbon dioxide was trapped in ten ml of phenethylamine phosphor solution (Woeller, 1961). The trapped CO_2 was then prepared for ^{14}C assay. Several other minor modifications to the procedure, involving reaction times and quantity of reactants, were also made. (See Appendix for procedure.)

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[1]Varian Aerograph, P.O. Box 517, Walnut Creek, California.

Radioactive assay of samples. In all cases, activity of the samples was determined in a Packard liquid scintillation counter. In the first series of experiments, when ten ml of 0.5 N NaOH was used as a trapping solution for CO_2, 0.1 ml and 1.0 ml aliquots of the NaOH was pipetted into scintillation counting vials containing 9.9 ml and 9.0 ml, respectively, of phosphor solution (Keith, 1963). (See Appendix for composition.) The vials were then chilled and dark adapted in the scintillation counter and counted three times for ten minutes each. Activity of the samples was determined by correcting the observed counts for efficiency, quenching and sample size and reported as disintegrations per minute (dpm).

In the second series of experiments, CO_2 was trapped directly into ten ml of phenethylamine phosphor solution (Woeller, 1961). (See Appendix for composition.) The ten ml of phosphor was then transferred directly to a counting vial and counted and reported as before.

RESULTS AND DISCUSSION

Establishment of cultures and placing them in carbon balance and steady state. At the beginning of this investigation, much time was spent establishing the experimental subcultures and learning their characteristics. Equipment such as fermentation flasks with large volume (120 ml) manometers as depicted on Plate I had to be prepared as well as techniques developed for this study. Four experimental subcultures,

cultures A, B, C and D, were developed. Throughout the period of investigation, cultures B, C and D were maintained in identical manner and during tracer studies, each culture was always administered the same carbon-14-labeled benzoic acid substrate. That is, culture B was always fed benzoic-1-^{14}C acid; culture C, benzoic-4-^{14}C acid and culture D, benzoic-7-^{14}C acid. Culture A was kept in reserve. In addition, four large stock cultures were available.

Studies were made and records kept to insure that each culture was in carbon balance throughout the period of investigation. The cultures must be in carbon balance so that most of the substrate is returned as gas rather than used as cell building material. Table 1 depicts these cultures in a state of carbon balance during a 14 day period.

Table 1. Carbon balance study: benzoic acid as a substrate.

Culture	Substrate (mM)	Theoretical Yield (ml)	Actual Gas Yield (ml)	% Carbon Recovery
B	2.0	372	370	99.3
C	2.0	372	369	99.2
D	2.0	372	369	99.2

During this study, the cultures were allowed to partially deplete the available substrate. At intervals, each culture was fed a total of two millimoles of benzoic acid substrate over the 14 day period. At the end of the experiment, the cultures were again allowed to nearly deplete their substrate. As indicated,

they were able to evolve nearly 100% of the theoretical equivalent gas yield.[1]

Cultures were also maintained in an almost steady state of gas production during experiments. Several days prior to each experiment, cultures were given slightly more substrate than they could metabolize and thus forced to produce gas at a maximum steady rate as shown in Figure 1 and Table 4. (See Appendix for Table 4.) Since all cultures performed in like manner, culture C was selected to be representative.

Several conclusions can be drawn from these experimental cultures. A culture can produce gas at an almost steady rate and be forced to increase or decrease this rate by the amount and interval of feeding. The gas production of the three experimental cultures could be increased to 6.0 to 7.0 ml of gas per hour. Also noted, the per cent carbon recovery remains fairly constant throughout the period of increased gas production and once the available substrate is utilized, the carbon recovery approaches 100% of the theoretical yield. (See Appendix for Table 4.) This indicates that the bacterial numbers remain fairly constant and do not use the excess substrate in proliferation of cells once a culture is established; but rather, they adapt to increased gas production. Further, the cultures were able to maintain their relative rate of gas production after the culture liquor, including substrate, was removed and replenished with new Barker's

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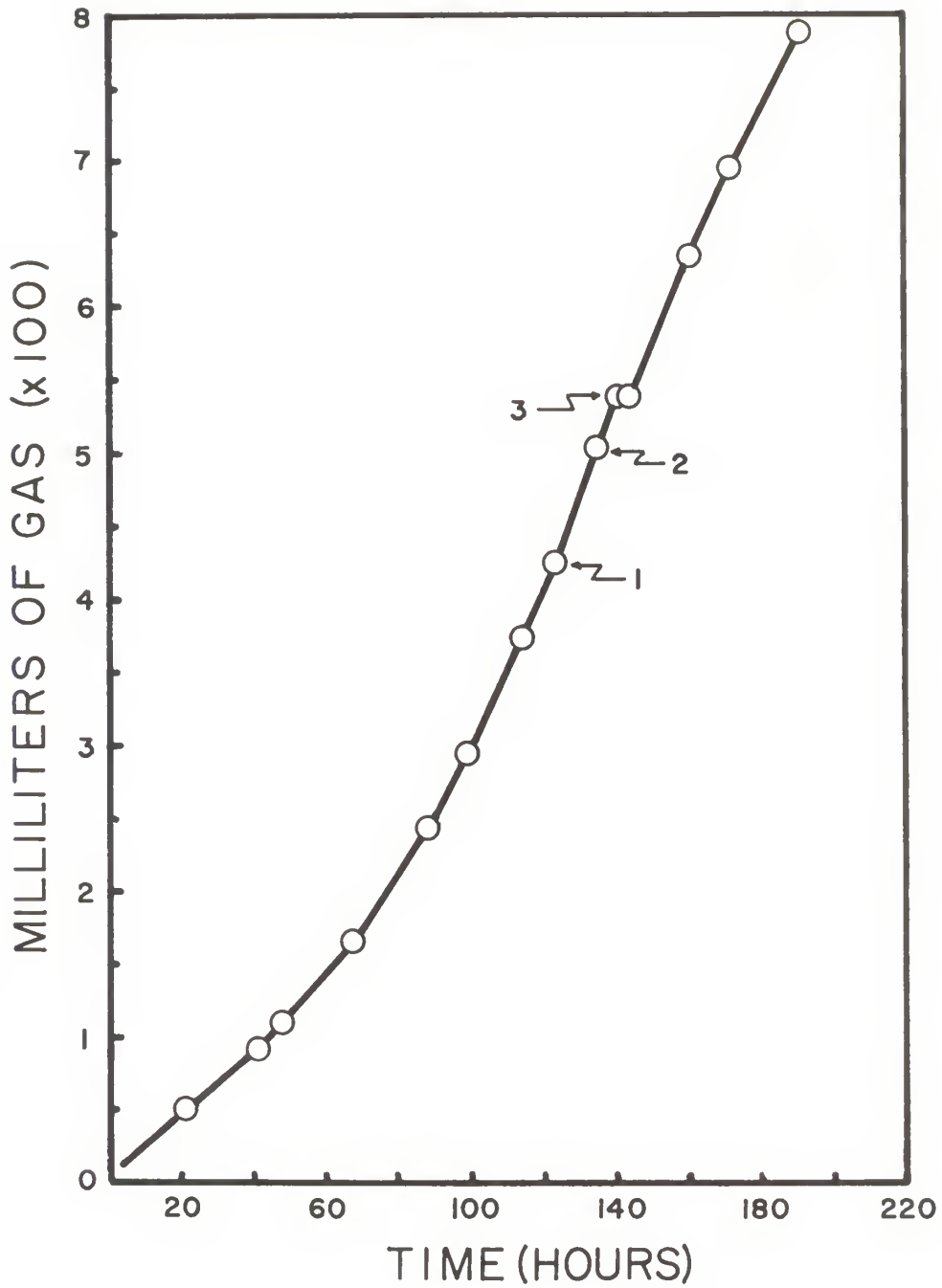
[1]One millimole of benzoic acid yields seven millimoles of gas. This is equivalent to about 186 ml of gas when corrected for temperature and altitude.

EXPLANATION OF FIGURE 1

This chart depicts gas production by culture C. Complete details are found in Table 4 in the Appendix.

1. Introduction of benzoic-4-^{14}C acid.
2. Introduction of nonlabeled sodium propionate.
3. Culture liquor completely removed and culture replenished and stabilized.


MILLILITERS OF GAS (x100)
TIME (HOURS)
0
1
2
3
4
5
6
7
8
20
60
100
140
180
220
1
2
3

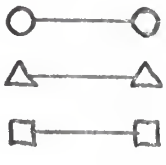
mineral salt solution. The shock of this treatment apparently did not affect the cultures adversely. Also, when the cultures were fed two millimoles of unlabeled neutralized propionic acid, a proposed intermediate used to entrap the ^{14}C by the method of isotopic dilution, there was no lag in gas production. This observation is in agreement with the theory of simultaneous adaptation (Stanier, 1947) and would indeed indicate that propionic acid was a possible intermediate of these benzoic acid adapted cultures as proposed by Roberts (1962).

Elimination rate of radioactivity from cultures. During each series of tracer experiments, all the culture liquor was removed from the cultures for analysis. It was then necessary to re-establish the cultures for future use. The major problem was the elimination of residual radioactivity. Figure 2 shows the rate of decrease of activity in each culture during the interval between experiments.

It is interesting and significant that cultures C and D always required a longer period of time to eliminate carbon-14 than did culture B. As already demonstrated by Fina and Fiskin (1960), carbon-1 of benzoic acid is converted almost entirely to methane while carbon-7 goes to carbon dioxide. Culture B, fed benzoic-1-^{14}C acid, naturally would rapidly eliminate the relatively insoluble $^{14}CH_4$ being produced while the $^{14}CO_2$ produced from culture D, fed benzoic-7-^{14}C acid, would tend to remain in the culture longer as carbonates. The similarity of the elimination rate of carbon-14 between cultures C and D would indicate that carbon-4 of benzoic acid becomes carbon

EXPLANATION OF FIGURE 2

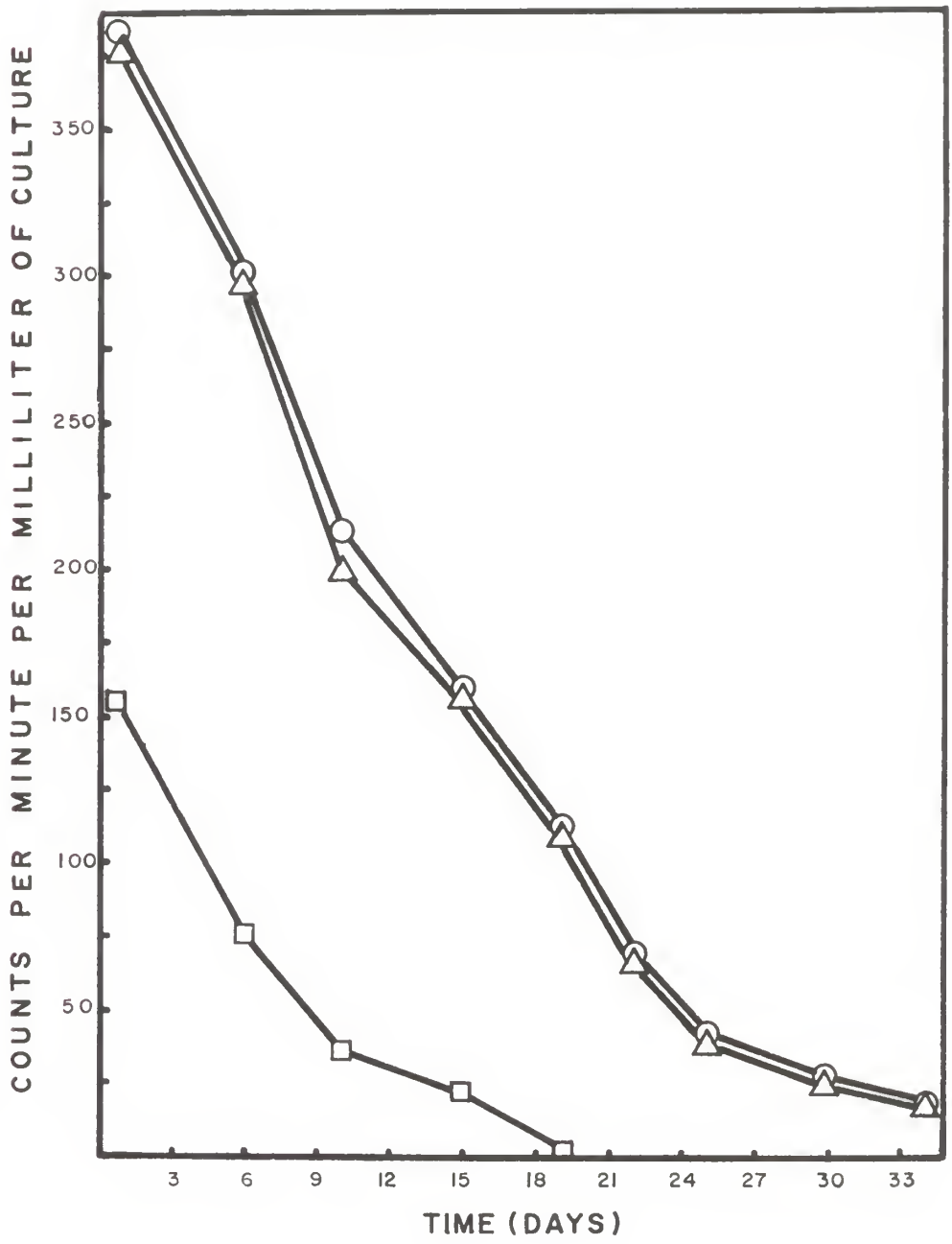
This chart depicts the elimination rate of radioactivity from the experimental cultures.



Culture fed benzoic-4-^{14}C acid.

Culture fed benzoic-7-^{14}C acid.

Culture fed benzoic-1-^{14}C acid.


COUNTS PER MINUTE PER MILLILITER OF CULTURE
350
300
250
200
150
100
50
3
6
9
12
15
18
21
24
27
30
33
TIME (DAYS)

dioxide as does carbon-7. Culture C was fed benzoic-4-^{14}C acid.

Fate of carbon-14 labeled benzoate species during benzoic acid utilization. Early in this study, attempts to isolate specific intermediates directly from the experimental subcultures proved fruitless. Gas chromatography and other analytical techniques were employed. Other workers have experienced similar difficulties attempting to isolate intermediates from methanogenic cultures regardless of the substrate used. Also, another major difficulty encountered by this investigator, and not as yet completely solved, was the separation of any free intermediate from the benzoic acid substrate present.

Because of the fleeting nature of any intermediates in the culture and the difficulties in isolating them, the isotopic dilution approach to the problem was used. Propionic acid was previously suggested as an intermediate by benzoic acid utilizing cultures (Roberts, 1962). The question was: is propionic acid a direct intermediate; and if so, which of the carbons of benzoic acid contributed to the C-3 fragment? The exact labeled position in the molecule was determined by subjecting the intermediate, obtained by the method of isotopic dilution, to Schmidt degradation as discussed in materials and methods. By the use of such a reaction, the isotopic concentration of each carbon in a fatty acid chain may be determined.

Before the fate of these labeled carbon atoms could be discovered by the above methods, it was essential to determine two things. First of all, it was necessary to determine the purity of the entrapping propionic acid as isolated from each

experimental culture. This was accomplished by gas liquid chromatography as discussed in materials and methods. It was repeatedly demonstrated that only the isolated propionic acid was present. This was necessary so that the results of the later degradation reactions would be conclusive. Any residual benzoic acid substrate was eliminated by a low column temperature.

It was next necessary to determine the adaptability and efficiency of the Phares-Schmidt degradation with various species of propionic and benzoic acids. No information was available concerning the effect of this reaction on benzoic acid. Results of this series of experiments are found in Table 2.

Table 2. Phares-Schmidt degradation. Fate of propionic acid and benzoic acid species labeled with carbon-14.

Compound Tested	Quantity of Isotope[1]	Average Disintegrations Per Minute[2]
Propionic-1-^{14}C Acid	0.5 uC	6.82×10^5
Propionic-2-^{14}C Acid	0.5 uC	0.0
Benzoic-1-^{14}C Acid	0.5 uC	0.0
Benzoic-7-^{14}C Acid	0.5 uC	6.53×10^5

[1]One-half a mM of benzoate with a sp. act. of 1 uC per mM.

[2]Average of two determinations adjusted for machine efficiency, quenching, sample size and calculated as disintegrations per minute above background.

The Phares-Schmidt degradation reaction, modified as described in materials and methods, was proved to be satisfactory for this study. Only the carboxyl of propionic acid and benzoic

acid was affected by the reaction. The ring was not ruptured, and there was no cross-contamination between molecular portions.

In the experiments that followed, it was established that carbon-4 of benzoate becomes the carboxyl carbon of the intermediate propionic acid. Culture liquor from each subculture was prepared for analysis as discussed; and the dried samples, from each culture, were tested for the fate of ^{14}C. Table 3 shows the combined results of two series of experiments.

Table 3. Fate of benzoic acid carbons as revealed by Phares-Schmidt degradation of propionate.

Culture	Labeled Substrate	$^{14}CO_2$ Trapping Solution	Disintegrations Per Minute[1]
B	ØCOOH-1-^{14}C	0.5 N NaOH	0
B	ØCOOH-1-^{14}C	Phenethylamine	57
C	ØCOOH-4-^{14}C	0.5 N NaOH	6,400
C	ØCOOH-4-^{14}C	Phenethylamine	14,600
D	ØCOOH-7-^{14}C	0.5 N NaOH	95,100

[1]Average of two determinations, adjusted for machine efficiency, quenching, sample size and calculated as disintegrations per minute above backgroud for the entire quantity of culture liquor. DPM in the CO_2 from the carboxyl of propionate.

Sodium hydroxide (0.5 N) was used as a CO_2 trapping agent in the first series of experiments. This proved a poor choice for several reasons: relatively small quantities of the labeled intermediate was present in the culture liquor, and it was necessary to assay only a small portion of the total NaOH for ^{14}C. (See materials and methods.) If more than 0.1 ml of

0.5 N NaOH was used, excessive quenching occurred. Thus, the level of activity above background counts was subject to considerable error.

For the next series of experiments, this problem was alleviated by using phenethylamine plus phosphors as a trapping solution (Woeller, 1961). The use of phenethylamine avoided the disadvantages of NaOH; and above all, the trapping solution in total was used as the counting solution. All the trapped $^{14}CO_2$ could be counted directly and thus avoid errors introduced by using aliquots. The above series of experiments showed conclusively that carbon-4 of benzoic acid becomes the carboxyl carbon of the entrapped propionic acid. Further, carbon-1 does not appear as a carboxyl carbon.

Until the initial attack on the ring of benzoic acid is positively known and other intermediates elucidated, any suggestion as to a possible anaerobic pathway or comparison to known aerobic pathways would be premature. However, with the understanding that it is only speculation, several deductions can be drawn from the information obtained from this investigation and past studies.

The similarity of elimination rates of ^{14}C between carbon-4 and carbon-7 of the experimental cultures suggests that carbon-4 is converted primarily to CO_2. Further, it was demonstrated that carbon-4 became the carboxyl carbon of the intermediate propionic acid which is in agreement with the above statement. Roberts (1962) showed that propionic acid did not, however, contain carbon-7 of benzoic acid. With these facts, one can speculate

that propionic acid does not arise indirectly by a carboxylation mechanism involving the carboxyl of benzoic acid; but rather, it arises directly after cleavage of the ring. Also, since carbon-4 persists as the carboxyl carbon of propionic acid, this supports the idea that ring cleavage occurs between carbons 1 and 2 or carbons 1 and 6, with the resulting seven carbon chain then degraded two carbons at a time leaving the C-3 fragment of propionic acid.

No research can be worthwhile unless it suggests additional areas of study. Attempts should be made to isolate additional possible intermediates, such as cyclohexanecarboxylic acid, heptonoic acid and valeric acid, by methods described in this study. Then by sequential degradation of these isolates, the exact disposition of the carbon atoms of benzoic acid in these intermediates could be ascertained. It is also suggested that large vat-type fermentors be developed to grow sufficient cells for cell free extract studies. Only by further research can the anaerobic aromatic pathway of these cultures be clearly established.

SUMMARY

This study was a continued investigation of the anaerobic utilization of benzoic acid by methanogenic cultures which were placed in a state of carbon balance and steady state. Data derived from this study led to the following conclusions:

1. Attempts to isolate intermediates directly from culture

liquor proved unsatisfactory.

2. Elimination rates of ^{14}C from different experimental cultures showed a close similarity between carbon-4 and carbon-7 of benzoic acid. This suggests that carbon-4 is converted primarily to CO_2.
3. Isotopic dilution techniques were used to establish the intermediate propionic acid. The fate of various labeled carbon atoms of benzoic acid in this intermediate was then determined by Phares-Schmidt degradation, as modified for this study.
4. It was demonstrated that carbon-4 of benzoic acid becomes the carboxyl carbon of the entrapped propionic acid.
5. It was further demonstrated that carbon-1 of benzoic acid does not appear as a carboxyl carbon.

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APPENDIX

Composition of Barker's mineral salt solutions (Barker, 1936) as modified by Fina et al., (1960).

Solution A		Solution B	
$CaCO_3$	6.33 gm	Na_2S	1.0 gm
K_2HPO_4	0.40 gm	Na_2CO_3	5.0 gm
NH_4Cl	1.00 gm	Distilled water	100 ml
$MgCl_2$	0.10 gm		
Tap water	1000 ml		

Solutions A and B are mixed in a ratio of 100 ml of solution A to three ml of solution B just prior to use. The sodium sulfide in solution B is used to lower the oxidation-reduction potential of the medium.

Composition of phosphor solution (Keith, 1963).

p-dioxane	1000 ml
POP	6 gm
POPOP	0.05 gm
Naphthalene	100 gm

Composition of phenethylamine phosphor solution (Woeller, 1961).

27 ml of redistilled phenethylamine[1]

27 ml of absolute methanol

500 mg 2, 5-diphenyloxazol (POP)

10 mg 1, 4-bis-2-(5-phenyloxazolyl) benzene (POPOP)

100 ml toluene

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[1]Matheson, Coleman and Bell Division, Cat. No. 6066; Price: $11.25 per kilogram.

Phares-Schmidt degradation procedures as modified for this study.

The Phares-Schmidt degradation involves the decarboxylation by use of the Schmidt reaction and the oxidation of the resulting amine to an acid of one less carbon atom. This allows the direct determination of the isotopic concentration associated with a particular position in the molecule. For the purpose of this study, only the decarboxylation step was required.

The apparatus for the degradation of the samples consisted of a reaction flask equipped with a sweep-tube, a scrubber containing 5% $KMnO_4$ in 1 N H_2SO_4 and a trap to collect CO_2. An inlet for CO_2 free air (Chen, 1957) was provided through the sweep-tube and a mercury check-valve allowed gas to escape after passing through the CO_2 trap.

The following procedure was used:

1. Cool reaction flask with contents to about 5° C and add approximately 1 ml of 100% H_2SO_4[1].
2. Dissolve sample completely by warming and shaking.
3. After recooling flask, add approximately 100 mg (1.54 mmoles) of sodium azide and again warm until the azide is nearly dissolved.
4. Connect the reaction flask to the traps and place it in a 35° C bath.
5. Allow the temperature of the bath to rise over a period of

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[1]For each mM of dried sample, add 1 ml of 100% H_2SO_4 prepared by dilution of one part C. P. fuming H_2SO_4, 20% excess SO_3, with three parts C. P. concentrated H_2SO_4.

30 minutes to 60-70° C.

6. Allow the reaction to continue for one hour at this temperature, then open the screw-clamp on the sweep-tube and sweep the system for 30 minutes with CO_2 free air.
7. Assay the trapped CO_2 for radioactivity.

Table 4. Carbon balance and steady state studies of culture C.

Hours	Benzoic Acid Substrate (mM) Fed	(Total)	Theoretical Equivalent Gas Yield (ml) Total Fed	Actual Gas Yield (ml)	ml/hr since last feeding	% Carbon Recovery
0.0	0.3	(0.3)	56.0	---	---	---
21.5	0.3	(0.6)	112.0	50.0	2.3	89.8
26.5				65.0		
41.5	0.3	(0.9)	166.0	93.0	2.1	83.5
47.5	0.5	(1.4)	259.0	110.0	2.8	66.3
53.5				135.0		
64.0				167.0		
67.5	0.5	(1.9)	352.0	167.0	3.3	67.8
74.5				205.0		
88.5	0.5	(2.4)	445.0	243.0	3.2	69.0
96.0				283.0		
98.5	0.5	(2.9)	538.0	293.0	5.0	65.8
111.5				362.0		
114.0	0.3	(3.2)	594.0	373.0	5.1	69.3
119.0				401.0		

Table 4. (continued)

Hours	Benzoic Acid Substrate (mM) Fed	(Total)	Theoretical Equivalent Gas Yield (ml) Total Fed	Actual Gas Yield (ml)	ml/hr since last feeding	% Carbon Recovery
123.5	0.5	(3.7)	687.0	426.0	5.6	71.9
126.5	0.3	(4.0)	743.0	446.0	6.7	65.0
135.5	0.3	(4.3)	799.0	503.0	6.3	67.9
141.5				539.0	6.0	67.6
All culture liquor removed for analysis and culture replenished and stabilized (2 hr.)						
0.0	1.0	(1.0)	186.0	---	---	---
17.5	0.5	(1.5)	279.0	94.0	5.4	50.5
28.5				154.0		
38.5	0.5	(2.0)	372.0	200.0	4.9	71.6
48.5				254.0		
64.0				317.0		
71.0	0.5	(2.5)	465.0	338.0	4.2	91.0

THE ANAEROBIC DECOMPOSITION OF BENZOIC ACID DURING METHANE FERMENTATION: EVIDENCE THAT CARBON FOUR OF BENZOIC ACID IS CONVERTED TO THE CARBOXYL OF THE INTERMEDIATE PROPIONIC ACID

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Studies of the anaerobic decomposition of benzoic acid during methane fermentation were undertaken to add information or further delineate a possible anaerobic aromatic pathway. For this investigation, an isolated model system comprised of cultures of methanogenic bacteria, established in all-glass fermentors, was used. Anaerobic conditions were always maintained.

At the beginning of the investigation, time was spent establishing the experimental cultures, learning their characteristics and developing equipment and techniques. During experiments, the cultures were placed in carbon balance and maintained in a steady state of gas production. Once established, they were able to evolve nearly 100% of the theoretical equivalent gas yield from the benzoic acid substrate with very little substrate used as cell building material.

After fruitless attempts to isolate intermediates directly from culture liquor, experiments were set up to study the fate of benzoic acid species labeled in either the 1, 4 or 7 positions with ^{14}C. Because of the fleeting nature of any intermediates in the cultures and the difficulties in isolating them, the isotopic dilution approach was used to establish the intermediate propionic acid. Propionic acid had previously been suggested as a possible intermediate. It was definitely established by this technique that propionic acid was an intermediate. Its purity was determined by gas liquid chromatography. Also, the exact labeled position in the molecule of the entrapped propionic acid intermediate was determined by the Phares-Schmidt degradation as modified for this study. It was conclusively demonstrated that

carbon-4 of benzoic acid becomes the carboxyl carbon of the intermediate propionic acid; and further, carbon-1 does not appear as a carboxyl carbon.

Other studies showed nearly identical elimination rates of carbon-7 and carbon-4. They required a much longer period of time to be eliminated from the culture than did carbon-1 which had previously been shown to be converted to methane. This strongly suggests that carbon-4 is converted primarily to CO_2 as is carbon-7. Other investigators had previously determined that carbon-7 was primarily converted to CO_2. This finding was in agreement with the fact that carbon-4 is converted to the carboxyl of the intermediate propionic acid during the degradation of this substrate.